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A Novel Mode of Action of the Putative Sphingosine Kinase Inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) Thiazole (SKI II): Induction of Lysosomal Sphingosine Kinase 1 Degradation

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Key Words

SKI II • Sphingosine kinase 1 • Protein degradation • Lysosome • Chloroquine • Cathepsin B

Abstract

Background: Sphingosine kinase 1 (SK1) is a key enzyme in the generation of sphingosine 1-phosphate (S1P) which critically regulates a variety of important cell responses such as proliferation and migration. Therefore, inhibition of SK-1 has been suggested to be an attractive approach to treat tumor growth and metastasis formation. Results: We show here that the previously developed putative SK-1 inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II) displays an additional facet of action complementary to the known inhibition of enzymatic SK-1 activity. In various human cell lines including glomerular podocytes and mesangial cells, the human endothelial cell line EA.hy 926, and the lung cancer cell line NCI H358, SKI II reduced TGFβ- and TPA-stimulated cellular SK-1 activity by downregulating SK-1 protein expression without affecting SK-1 mRNA expression. By using cycloheximide to block the de novo protein synthesis, the protein expression of SK-1 under untreated conditions was stable over 24h. Under SKI II treatment, the half-live drastically decreased to approximately 0.8h. Mechanistically, this degradation occurred through a lysosomal pathway and involved cathepsin B since the general lysosomal inhibitor chloroquine and the specific cathepsin B inhibitor CA-074ME were able to reverse the effect of SKI II. Surprisingly, in vitro SK-1 activity assays revealed only a very weak direct inhibitory effect of SKI II on SK-1 overexpressed HEK293 cell lysates. Conclusion: These data show for the first time that the previously developed SK inhibitor SKI II hardly inhibits SK-1 directly but rather acts by triggering the lysosomal degradation of SK-1 in various cell types. This finding discloses a new mode of action of SKI II and strongly suggests that additional direct targets of SKI II may exist other than SK-1.

Introduction

Sphingosine 1-phosphate (S1P) has attracted a lot of interest in the last years due to its key regulatory role in various physiological and pathophysiological processes such as cell proliferation and differentiation, migration,

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angiogenesis, and tumour development [1-4]. S1P is generated by sphingosine kinases (SK) which includes the two subtypes SK-1 and SK-2 [5]. Although both subtypes catalyze the same reaction, it remains unclear whether they have overlapping or diverging cellular functions [5]. Accumulating evidence now suggests a key role for SK-1 in cell proliferation and migration implicating a role of this subtype in tumour growth and metastasis formation [4-7]. Additionally, the overexpression of SK-1 in mouse fibroblasts leads to foci formation in soft agar thus, suggesting that the *SPHK1* gene may act as an oncogene [8]. Consistently, SK-1 was found to be overexpressed in various forms of solid tumours compared to healthy tissue [9, 10]. In contrast, SK-2 has rather been attributed a pro-apoptotic role due to its BH3 domain which may interact with the Bcl2 family member Bcl-XL and neutralize its anti-apoptotic function finally leading to a pro-apoptotic response [11]. Also, a differential subcellular localization of SK-1 and SK-2 suggests opposite functions. The SK-1 is mainly localized in the cytosol of cells but can translocate to the plasma membrane [12] or to the nucleus upon activation [13]. Furthermore, it was shown that the SK-1 contains two nuclear export signal sequences (NES) [14]. When nuclear export was blocked by the export inhibitor leptomycin B, SK-1 accumulated in the nucleus [14]. In contrast, SK-2 is rather localized in the nucleus and may translocate to the cytosol under certain conditions [15, 16].

Based on the suggested involvement of SK-1 in cell proliferation and migration, new SK-1 inhibitors as potentially attractive cancer therapeutics were developed. To date, only few SK inhibitors have been described such as the sphingosine analogues N,N-dimethylsphingosine (DMS) and D/L-*threo*-dihydrosphingosine (DHS, known as safingol). These compounds were reported to competitively inhibit SK-1 activity [17-19]. However, they were originally described as protein kinase C inhibitors and shown to block this enzyme in vitro with equal or even higher potency [20, 21]. Still, controversial data exist on the inhibition of PKC by DMS, since in cell culture experiments, DMS did not reduce phorbol ester-stimulated PKC activity or translocation to the membrane [22].

More recently, a series of more selective inhibitors of human SK were developed and validated [9]. Although not specifically noted in that report, the human SK that was used for the screening was identical to the later specified subtype SK-1. Among the screened compounds, the 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II) showed the highest selectivity towards SK-1 with no inhibition of either protein kinase C-α, the classical mitogen-activated protein kinase p42-MAPK/ERK2, or the phosphatidylinositol 3-kinase [9]. The IC_{50} value of SKI II for SK-1 in vitro was indicated as 500 ± 300nM. In cell culture studies using the breast cancer cell line MDA-MB-231, SKI II reduced cellular S1P levels by approx. 75% at a concentration of 20 g/ml which corresponds to 66 μM [9].

In this study, we show that in various cell types, including cells of mesenchymal, endothelial, and epithelial origin, SKI II exerted an additional mechanism of action by downregulating agonist-induced SK-1 protein expression instead of directly inhibiting SK-1 catalytic activity. This effect occurred through triggering a lysosomal degradation route which involved the lysosomal protease cathepsin B.

Materials and Methods

Chemicals

Transforming growth factor-β_2 was from R&D Systems (Wiesbaden, Germany); cycloheximide, chloroquine, and lactacystin were from Sigma Aldrich Fine Chemicals (Deisenhofen, Germany); TPA, SKI II, and CA-074ME were from Merck Biosciences, Schwalbach, Germany; [^{32}P]-γATP (specific activity, >5000Ci/mmol) was from Hartmann Analytic GmbH (Braunschweig, Germany). The GAPDH antibody (V-18; sc-20357) was from Santa Cruz Biotechnology (Heidelberg, Germany); the human SK-1 antibody was generated and characterized as previously described [23, 24].

Cell culture

The human immortalized podocyte cell line was kindly provided by Hermann Pavenstaedt (University of Münster, Germany) and cultured as previously described [25]. The human endothelial cell line (EA.hy 926) was provided by Dr. Edgell (NC, US) [26]. The human non-small lung cancer cell line NCI-H358 was obtained from the American Tissue Culture Collection. Primary cultures of human mesangial cells were provided by Prof. Radeke, Frankfurt, Germany. Prior to stimulation cells were synchronized by incubation for 16h in Dulbecco's modified Eagle's medium (DMEM) including 0.1 mg/ml of fatty acid-free bovine serum albumin.

Cell transfections

HEK293 cells were transfected using LipofectAMINE according to the manufacturer's instructions. For transfections 1 μg of either pcDNA3.1 vector alone or vector containing full-length cDNA of human SK-1 or SK-2 was used. 48h post-transfection, cells were taken for further stimulation or cell lysis.

Western blot analysis

Stimulated cells were homogenized in lysis buffer [27] and centrifuged for 10min at 14'000 x g. The supernatant was taken for protein determination. 30 μg of protein were sepa-

rated on SDS-PAGE, transferred to nitrocellulose membrane and Western blot analysis was performed as previously described [23, 24] using antibodies as indicated in the figure legends.

Sphingosine kinase activity assay

In vitro kinase reactions were performed exactly as previously described [23, 24].

Quantitative RealTime PCR (SybrGreen)

Real-time PCR was performed using a BioRad iQ iCycler Detection System. Primer sequences were as follows: human SK-1 (accession number: NM_021972): forward: GGG CTT CAT TGC TGA TGT GGA C; reverse: TGC CTG CCA TTA CAA CTG TCC; human GAPDH (accession number: NM_002046): forward: GCT CTC TGC TCC TCC TGT TC; reverse: CGC CCA ATA CGA CCA AAT CC. IQ™5 Optical Optical System Software (Version 2.0) was used to analyze real time and endpoint fluorescence.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test for multiple comparisons (GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, USA).

Results

SKI II inhibits agonist-upregulated SK-1 activity by downregulating SK-1 protein expression in various cell types

Stimulation of human glomerular epithelial cells (podocytes) with transforming growth factor-β_2 (TGFβ) led to a pronounced upregulation of SK-1 protein expression as detected by Western blot analysis (Fig. 2A) which is confirmatory to our previous observations [25]. The increased SK-1 protein expression was accompanied by an increased activity of SK-1 when measured in an in vitro kinase assay using sphingosine as a substrate (Fig. 2B). In the presence of the recently developed and commercially available SK inhibitor SKI II [9], the cellular SK-1 activity was reduced (Fig. 2B). Since SKI II treatment also downregulated SK-1 protein expression (Fig. 2A) these data suggest that SKI II may reduce cellular SK-1 activity by downregulation of the protein rather than by directly inhibiting the catalytic activity of the enzyme.

In contrast, the levels of cellular SK-2 protein expression and cellular activity were not affected by SKI II (data not shown). However, it should be noted that SK-2 mRNA and protein expressions were very low in podocytes when compared to SK-1 mRNA and protein expression. Also, basal SK-2 activity was

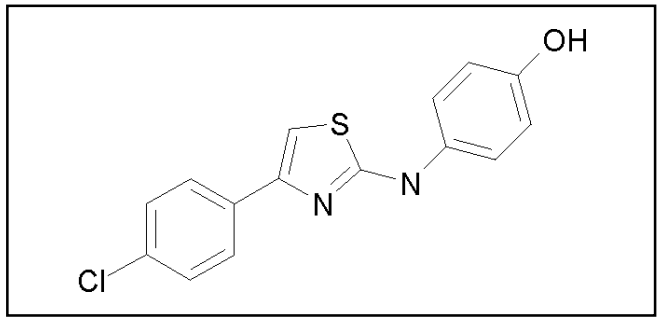

Fig. 1. Chemical structure of 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II).

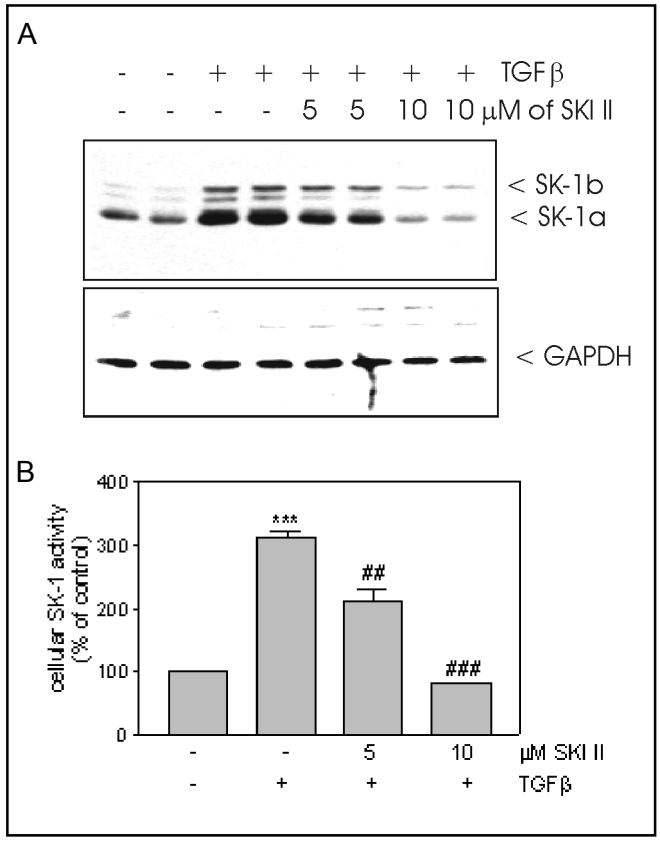

Fig. 2. Effect of SKI II on TGFβ-induced SK-1 activity and protein expression in human podocytes. Human podocytes were pretreated for 30min with either vehicle (-) or the indicated concentrations of the SK-1 inhibitor SKI II (in μM) prior to stimulation for 24h with TGFβ (5ng/ml; +). Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis (A) using specific antibodies against human SK-1 (A, upper panel) at a dilution of 1:1000, or GAPDH (A, lower panel) at a dilution of 1:2000, or taken for in-vitro SK-1 activity assays (B) as described in the Methods Section. The data in B are expressed as % of unstimulated controls of SK-1 activity and are means ± S.D. (n=3), ***$p<0.001$ considered statistically significant when compared to the unstimulated control values; ##$p<0.01$, ###$p<0.001$ when compared to the TGFβ-stimulated values.

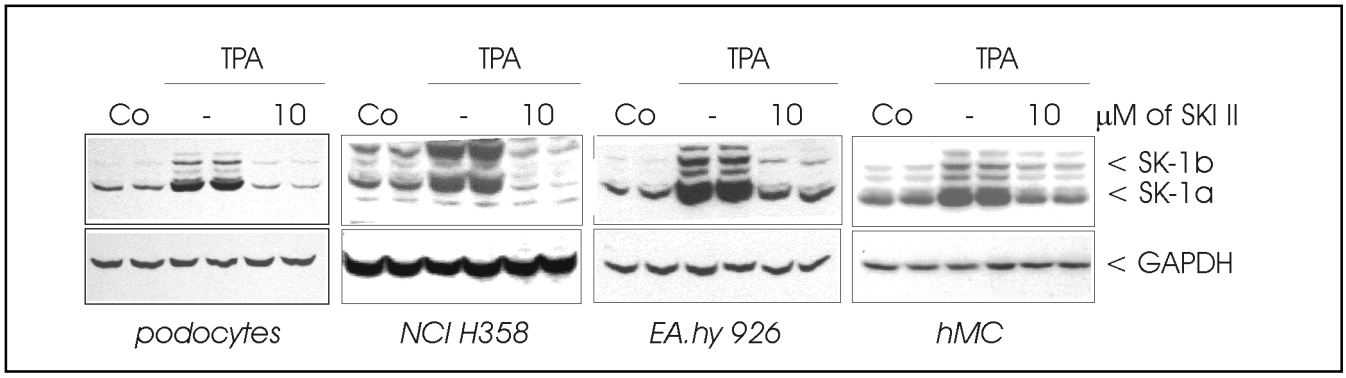

Fig. 3. Effect of SKI II on TPA-induced SK-1 protein expression in human podocytes, endothelial cells, lung cancer cells, and mesangial cells. Podocytes, NCI H358 cells, EA.hy 926 cells, and human mesangial cells (hMC) were pretreated for 30min with either vehicle (-) or the SK-1 inhibitor SKI II (10 µM) prior to stimulation for 24h with TPA (200 nM). Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using specific antibodies against human SK-1 (upper panels) at a dilution of 1:1000, or GAPDH (lower panels) at a dilution of 1:2000. Data are representative of 4 independent experiments giving similar results.

20-fold lower than basal SK-1 activity [25].

To see whether the observed effect of SKI II is specific for podocytes and for TGFβ stimulation, we further investigated other cell types and also used an additional stimulus to upregulate SK-1. As seen in Fig. 3, a similar reducing potential of SKI II was observed for TPA-upregulated SK-1 protein expression in either human podocytes, the human lung cancer cell line NCI H358, or the human endothelial cell line EA.hy 926. Also in cells of mesenchymal origin such as in primary cultures of human renal mesangial cells, SKI II downregulated TPA-induced SK-1 protein expression (Fig. 3).

SKI II inhibits TGFβ-induced SK-1 protein expression by triggering its lysosomal degradation

We further investigated whether the SKI II-triggered reduction of SK-1 protein expression was due to an inhibition of SK-1 gene transcription. To this end, mRNA expression of SK-1 was determined by quantitative PCR analysis. SKI II did not affect either TGFβ- or TPA-stimulated SK-1 mRNA steady-state levels (Fig. 4) indicating that the observed effect on protein expression might be due to a posttranscriptional effect, for example on SK-1 protein degradation. To address this point, podocytes were first stimulated for 16h with TGFβ to upregulate SK-1 protein expression. Cells were then treated with cycloheximide to block *de novo* protein synthesis and further incubated for up to 24 h in the absence or presence of SKI II. As seen in Fig. 5, the induced SK-1 protein was extremely stable and even after 24h, there was virtually no change in SK-1 protein expression level. Due to this high stability, a half-live could not be calculated. Strikingly, in the presence of SKI II

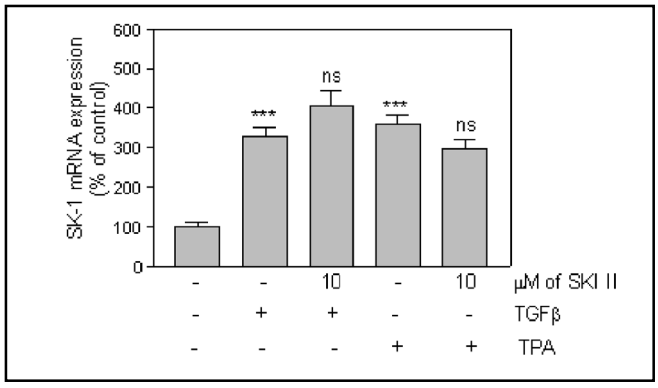

Fig. 4. Effect of SKI II on TGFβ– and TPA-induced SK-1 mRNA expression in human podocytes. Podocytes were pretreated for 30min with either vehicle (-) or 10 µM SKI II prior to stimulation for 4h with vehicle (-, control), TGFβ (5ng/ml) or TPA (200nM). Thereafter, RNA was extracted and used for quantitative PCR analysis of human SK-1 and 18S RNA as described in the Methods Section. Results are expressed as % of control values and are means ± S.D. (n=3). ***p<0.001 considered statistically significant when compared to the unstimulated control values; ns, not significant when compared to the TGFβ- or TPA-stimulated values.

the SK-1 protein levels dropped rapidly and reached basal levels after 2-4h. The half-live of SK-1 in the presence of SKI II was calculated to be approximately 0.8h.

In a next step, the mechanism of SK-1 degradation by SKI II was investigated. To this end, cells were pretreated with TGFβ followed by cycloheximide and then further incubated with SKI II in the presence of either the proteasomal inhibitor lactacystin [28] or the lysosomal inhibitor chloroquine [29]. As seen in Fig. 6, the SKI II-mediated effect on SK-1 degradation was partially recovered in the presence of chloroquine but not by

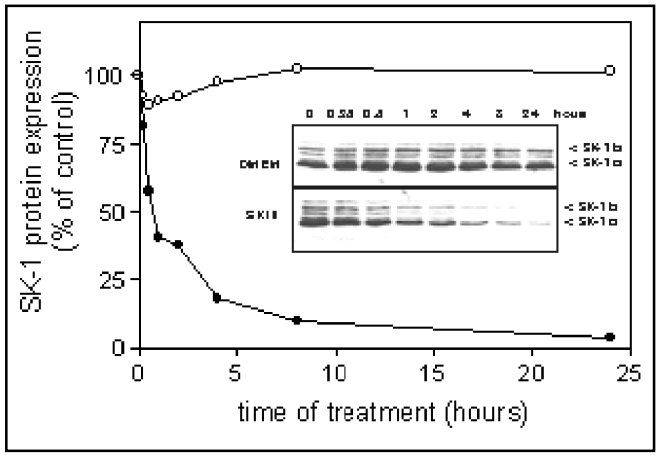

Fig. 5. Effect of SKI II on SK-1 protein degradation. Podocytes were stimulated for 24h with TGFβ (5 ng/ml) to maximally upregulate SK-1 protein expression. Then, cells were treated with cycloheximide for 30min to block de-novo protein synthesis. This time point was set to zero and cells were further incubated for up to 24h in the absence of SKI II (DMEM, open circles) or in the presence of SKI II (10 μM, closed circles). Thereafter, cell lysates were separated by SDS-PAGE, proteins were transferred to nitrocellulose and subjected to Western blot analysis using an antibody against human SK-1 at a dilution of 1:1000 (inset). Bands corresponding to SK-1a were densitometrically evaluated and plotted in a graph to show the degradation rate of SK-1a. The half-life of SK-1 in DMEM was not possible to calculate because the protein was stable over the 24h observation period; the half-life upon SKI II treatment was approximately 0.8h.

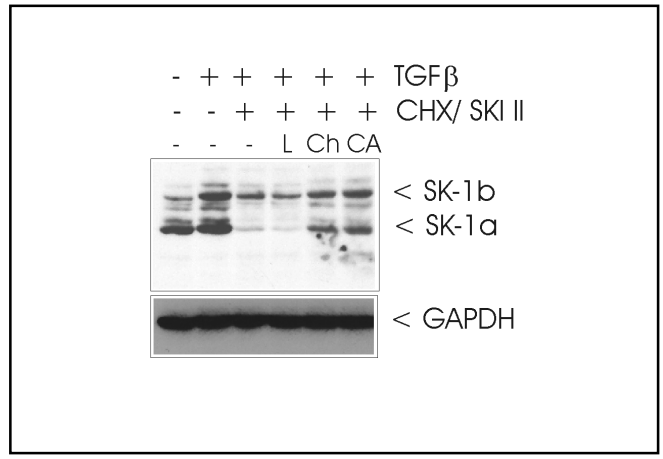

Fig. 6. Effect of proteasomal and lysosomal inhibitors on SKI II-triggered SK-1 protein degradation in human podocytes. Podocytes were pretreated for 16h with either vehicle (-) or 5 ng/ml TGFβ (+). Then, cells were treated with cycloheximide (5 μg/ml) for 30 min, and further incubated for 24h with SKI II (10 μM) in the absence or presence of either lactacystin (L, 10 μM), chloroquine (Ch, 50 μM), or the cathepsin B inhibitor CA 074-ME (CA, 10 μM). Thereafter, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose and subjected to Western blot analysis using specific antibodies against human SK-1 (upper panel) at a dilution of 1:1000, or GAPDH (lower panel) at a dilution of 1:2000. Data are representative of 3 independent experiments giving similar results.

lactacystin, indicating that SKI II triggers a lysosomal degradation route of SK-1. A strong reversal was also seen in the presence of the lysosomotropic agent ammonium chloride (data not shown). Furthermore, the specific cathepsin B inhibitor CA-074ME [30] also blocked the degradation of SK-1 (Fig. 6).

SKI II has only a weak direct inhibitory effect on SK-1 and SK-2 activities in vitro

Previously, it had been shown by French et al. [9] that SKI II potently inhibited the activity of purified E.coli-expressed human SK-1 *in vitro* with an IC_{50} of 500nM. To see whether SKI II also directly inhibited human SKs expressed in eukaryotic cells, the full length cDNA of human SK-1 and SK-2 were transiently overexpressed in human embryonic kidney epithelial cells (HEK) 293 cells and protein lysates were taken and treated *in vitro* with SKI II and subsequently subjected to activity assays. Surprisingly and in contrast to French et al. (9), we found only a minor effect of SKI II on SK-1 activity (Fig. 7, left panel). At 20 μM of SKI II, only 13% inhibition of

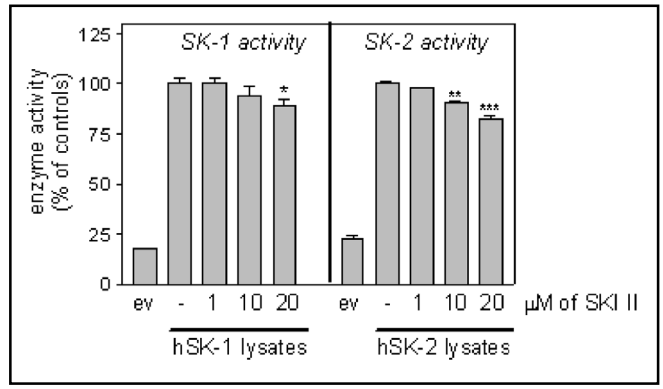

Fig. 7. *In vitro* effect of SKI II on human SK-1 and SK-2 activities. HEK293 cells were transiently transfected with either the empty vector pcDNA3 (ev) or with full-length human SK-1 (left panel) or SK-2 (right panel) as described in the Methods Section. Thereafter equal amounts of cell lysates were incubated in vitro with the indicated concentrations of SKI II and subsequently taken for *in vitro* SK-1 or SK-2 activity determination. Results are expressed as % of controls and are means ± S.D. (n=3); *p<0.05, **p<0.01, ***p<0.001 considered statistically significant when compared to the respective control values without inhibitor.

enzymatic activity was obtained. From these data an IC_{50} was extrapolated to be approximately 78 μM. Additionally, overexpressed human SK-2 was also tested. Also for this enzyme, only a minor effect of SKI II was seen (Fig. 7, right panel) although SKI II was slightly more active on SK-2 when compared to SK-1. At 20 μM of SKI II, a 23% inhibition of SK-2 was seen. An extrapolated IC_{50} of 45 μM was obtained.

Discussion

In this study, we show for the first time that the previously reported catalytic inhibitor of SK-1, SKI II, exerts an additional novel mode of action, and in various cell types, is able to down-regulate agonist-induced SK-1 protein expression rather than directly inhibiting catalytic SK-1 activity. Previously, French et al. [9] showed that SKI II is a rather selective SK-1 inhibitor when compared to other kinases such as PKC-α, PI-3K and ERK with an IC_{50} of 500 nM [9]. However, we could not see this potent inhibitory effect on human SK-1 in *in vitro* activity assays. Extrapolated from the weak inhibitory effect seen on SK-1 we determined an IC_{50} of 78 μM. Thus, in our hands the inhibitor was 150-fold less active than described. The reason for this striking discrepancy remains unclear. It may be speculated that the origin of the enzyme and/or its quality after purification is important. Indeed, the enzyme origin differed between the two studies. French et al. [9] used human SK-1 that had been coupled to glutathione S-transferase (GST) and expressed in *E.coli*, whereas in our study either human SK-1 cDNA overexpressed in HEK293 cells, or endogenous human SK-1 upregulated in EA.hy 926 cells after TPA stimulation (data not shown) were used. These latter sources of SK-1 were far less sensitive to SKI II than E.coli produced human SK-1.

Mechanistically, we identified the lysosome as one key organelle involved in SKI II-mediated SK-1 degradation. This is stressed by the findings that: (i) two general inhibitors of the lysosome such as chloroquine (Fig. 6) or ammonium chloride (data not shown) and, (ii) also a specific inhibitor of the lysosomal protease cathepsin B (Fig. 6), reversed the SKI II-triggered SK-1 degradation. In contrast, the proteasome was not involved in the SK-1 degradation since lactacystin had no effect of SKI II-triggered SK-1 degradation (Fig. 6).

Recently, Taha et al. [31] reported that in the breast cancer cell line MCF-7, tumor necrosis factor α (TNFα) triggered a time- and dose-dependent down-regulation of SK-1 which was abolished by depletion of cathepsin B by siRNA transfection. Furthermore, these authors also showed that cathepsin B can directly cleave SK-1 *in vitro* at two sites, His^{122} and Arg^{199}, leading to the appearance of two major fragments of 30kDa and 21kDa [32]. Interestingly, in our study using SKI II to trigger SK-1 degradation in various cell types, the two smaller fragments did not show up in Western blot analyses although the antibody recognizes an epitope at the C-terminus of human SK-1 and therefore should theoretically also detect the fragments. It may be speculated that these fragments are further rapidly degraded in cells and therefore escaped detection.

Cathepsins exist as a family of acidic proteases including many subtypes that are either cysteine proteases, such as cathepsins B, L, S, V, C, F, K, X, and H, or aspartic proteases, such as cathepsin D, or serine proteases such as cathepsin G [33, 34]. They are mainly localized in lysosomes where they find their optimal pH for activity and where they participate in lysosomal protein degradation. However, it is known that many apoptotic stimuli affect the lysosomes and increase lysosomal permeability resulting in the release of cathepsins into the cytosol where they can degrade various cytosolic proteins and enzymes [33, 34].

The exact mechanism by which SKI II triggers lysosomal degradation of SK-1 and whether this is a SK-1 specific effect or a more general effect also affecting other proteins determined for lysosomal degradation remains unclear. A series of other proteins, including membrane receptors such as the TGFβ receptors which are known to be internalised and degraded by the lysosomes [35, 36] were investigated but we could not see an accelerated degradation of these proteins (data not shown). Still, we can not exclude that other proteins are affected.

Furthermore, the well known apoptotic messenger ceramide was previously shown to directly bind to lysosomal cathepsin D which is activated upon ceramide binding [37]. However, since much higher concentrations of SKI II are needed to inhibit SK-1 activity than to see a degradation of SK-1 protein, it is unlikely that the effect was mediated by an accumulation of ceramide which in turn could activate cathepsins and degrade SK-1. Clearly, further studies are needed to address the details in SK-1 degradation triggered by SKI II.

In summary, our data have shown that in various human cell types, SKI II acts by stimulating SK-1 protein degradation and consequently by this mechanism reduces SK-1 activity rather than by directly inhibiting the

enzymatic activity as described by French et al. [9]. This degradation of SK-1 occurred by a lysosomal route involving cathepsin B and was triggered by SKI II. Thus, the use of SKI II causes a pronounced and irreversible inhibition of SK-1 by a posttranslational mechanism. This multitude of actions of a drug is not uncommon and may not preclude its usefulness in therapy. Moreover it proves again that specific inhibitors do not always give satisfactory answers because many of these agents are actually not highly specific and do not entirely inhibit the target enzyme. Nevertheless such compounds undoubtedly will provide therapeutically valuable information.

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